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**Citation for published version:**

Martins da Silva, SJ, Bayne, RAL, Cambray, N, Hartley, PS, McNeilly, AS & Anderson, RA 2004, 'Expression of activin subunits and receptors in the developing human ovary: activin A promotes germ cell survival and proliferation before primordial follicle formation', *Developmental Biology*, vol. 266, no. 2, pp. 334-45. <https://doi.org/10.1016/j.ydbio.2003.10.030>

**Digital Object Identifier (DOI):**

[10.1016/j.ydbio.2003.10.030](https://doi.org/10.1016/j.ydbio.2003.10.030)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Publisher's PDF, also known as Version of record

**Published In:**

Developmental Biology

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## Expression of activin subunits and receptors in the developing human ovary: activin A promotes germ cell survival and proliferation before primordial follicle formation

S.J. Martins da Silva, R.A.L. Bayne, N. Cambray, P.S. Hartley,  
A.S. McNeilly, and R.A. Anderson\*

*MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, University of Edinburgh, Edinburgh EH16 4SB, UK*

Received for publication 11 June 2003, revised 20 October 2003, accepted 22 October 2003

### Abstract

The formation of the essential functional unit of the ovary, the primordial follicle, occurs during fetal life in humans. Factors regulating oogonial proliferation and interaction with somatic cells before primordial follicle formation are largely unknown. We have investigated the expression, localisation and functional effects of activin and its receptors in the human fetal ovary at 14–21 weeks gestation. Expression of mRNA for the activin  $\beta$ A and  $\beta$ B subunits and the activin receptors ActRIIA and ActRIIB was demonstrated by RT-PCR. Expression of  $\beta$ A mRNA increased 2-fold across the gestational range examined. Activin subunits and receptors were localised by immunohistochemistry. The  $\beta$ A subunit was expressed by oogonia, and the  $\beta$ B subunit and activin receptors were expressed by both oogonia and somatic cells.  $\beta$ A expression was increased in larger oogonia at later gestations, but was low in oocytes within newly formed primordial follicles. Treatment of ovary fragments with activin A in vitro increased both the number of oogonia present and oogonial proliferation, as detected by bromodeoxyuridine (BrdU) incorporation. These data indicate that activin may be involved in the autocrine and paracrine regulation of germ cell proliferation in the human ovary during the crucial period of development leading up to primordial follicle formation. Crown Copyright © 2003 Published by Elsevier Inc. All rights reserved.

**Keywords:** Activin; Fetal ovary; Oocyte; Primordial follicle; Growth factor

### Introduction

Germ cell survival is key to the reproductive life span of the ovary and is determined by the continuing presence of oocytes within primordial follicles. These are formed during fetal life in the human. The number of follicles formed is dependent upon the balance between early germ cell proliferation and loss, as well as the interaction between oocyte and somatic cells (Hirshfield, 1991; McLaren, 1991). The pathways regulating this crucial process are largely unknown, although several genes necessary for the formation of a normal complement of primordial follicles have been identified (Amleh and Dean, 2002; Matzuk, 2000). During the period leading up to primordial follicle formation there is

massive oogonial mitotic proliferation, both during and after migration from the yolk sac to the nephrogonadoblastic ridge. The oogonia associate with somatic cells resulting in syncytial nests of germ cells intermingled with pre-granulosa cells and surrounded by a meshwork of ovarian stroma (Byskov, 1986; Pepling and Spradling, 2001; Sawyer et al., 2002). The germ cells subsequently enter meiosis only to arrest at diplotene of the first meiotic division. By mid-gestation, the human fetal ovary contains its maximal number of germ cells. This peak is followed by widespread germ cell loss, by apoptosis, (De Pol et al., 1997; Vaskivuo et al., 2001) so that less than 20% survive by the time of birth (Baker, 1963). Primordial follicles are formed as oocytes become individually surrounded by somatic cells (Hirshfield, 1991) and it is this interaction that has been identified as the event that determines oocyte survival, and presumably protection against apoptosis. A number of locally derived growth factors have been identified to be crucial to germ cell survival at early developmental stages, such as BMP-4 (Fujiwara et al., 2001) and kit ligand (Godin et al., 1991;

\* Corresponding author. MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, University of Edinburgh Chancellors' Building, 49 Little France Crescent, Old Dalkeith Road, Edinburgh, Scotland, EH16 4SB, UK. Fax: +44-131-2426231.

E-mail address: [r.a.anderson@hrs.u.mrc.ac.uk](mailto:r.a.anderson@hrs.u.mrc.ac.uk) (R.A. Anderson).

Manova et al., 1990), and following primordial follicle formation, such as growth and differentiation factor 9 (GDF-9) (Dong et al., 1996). However, little information is available regarding factors regulating oögonial proliferation and primordial follicle formation, particularly in human.

Activins and inhibins are members of the transforming growth factor  $\beta$  (TGF $\beta$ ) family. The component  $\alpha$  and  $\beta$  subunits can be combined into biologically active  $\alpha\beta$  inhibin or  $\beta\beta$  activin dimers with generally opposing functional effects, and are produced in both gonad and pituitary (Burger and Igarashi, 1988; Chen, 1993; Mather et al., 1997). Follistatin is a structurally unrelated monomeric protein that irreversibly binds activin and neutralises its biological effects (Nakamura et al., 1990; Schneyer et al., 1994). Members of the TGF superfamily can influence many facets of cell lineage and activity including proliferation, differentiation, adhesion, motility and apoptosis. Many are involved in embryonic development as well as adult tissue homeostasis (Mishina et al., 1999; Padgett and Patterson, 2001). The activins, together with other members of the TGF $\beta$  superfamily, signal through membrane-bound serine-threonine kinase receptors. They bind to a type II receptor (ActRIIA or ActRIIB), which recruits and phosphorylates a type I receptor with subsequent modulation of gene expression via Smad protein activation (Massagué, 1998). Several type I receptors, termed activin receptor-like kinases (ALKs) have been identified. ALK4 (ActRIB) is believed to be the preferential activin receptor, whereas ALK2 (ActRIA) preferentially mediates BMP and anti-Müllerian hormone signalling. Whilst the Smad proteins are the central elements in the activin receptor signalling pathway, other signalling pathways may mediate at least some of the diverse biological responses of the TGF $\beta$  superfamily, including mitogen-activated protein kinase (MAPK) pathways (Massague, 2000; Mulder, 2000). The initial steps coupling the activin type II serine-threonine kinase receptor to MAPK are yet to be defined, although signalling via TGF $\beta$ -activated kinase 1 (TAK1) results in activation of stress-activated p38 and transcription factor ATF2, which can interact with Smad3 and Smad4 to mediate transcription (Cocolakis et al., 2001; Derynck et al., 2001).

A substantial body of evidence has accrued that activins and inhibins are regulatory factors in the adult ovary involved in a wide range of functions, including folliculogenesis, granulosa and theca cell proliferation, and steroidogenesis (Findlay et al., 2002; Knight and Glister, 2001; Mizunuma et al., 1999) as well as oocyte maturation (Alak et al., 1998; Izadyar et al., 1998). Activin is expressed in the human gonad at very early stages of development (Harkness and Baird, 1997) and there are limited data indicating the expression of activin and inhibin in the developing human ovary following primordial follicle formation (Harkness and Baird, 1997; Rabinovici et al., 1991). However, no data are available regarding the presence of activin and its cognate receptors before primordial follicle formation in the human ovary. We have therefore explored

the expression and distribution of inhibin/activin subunits in the human fetal ovary during the period of oögonial proliferation leading up to primordial follicle formation. The localisation of activin receptors ALK4, ActRIIA and ActRIIB, the inhibin receptor betaglycan (Lewis et al., 2000) and the activin-binding protein follistatin has also been investigated to identify potential sites of action of these regulatory factors during this period of development. As oögonia in the mid-trimester human fetal ovary were found to express activin subunits and receptors, we have investigated the effect of activin A on oögonial survival and proliferation using an *in vitro* tissue culture model.

## Materials and methods

### *Tissues*

Human fetal ovaries were obtained following medical termination of pregnancy. Women gave consent according to national guidelines (Polkinghorne, 1989) and the study was approved by the Lothian Paediatrics/Reproductive Medicine Research Ethics Subcommittee. Termination of pregnancy was induced by treatment with mifepristone (200 mg orally) followed 48 h later by prostaglandin E1 analogue (Gemeprost; Beacon Pharmaceuticals, Tunbridge Wells, UK) 1 mg three hourly per vaginum. None of the terminations were for reasons of fetal abnormality, and all fetuses appeared morphologically normal. Gestational age was determined by ultrasound examination before termination and confirmed by subsequent direct measurement of foot length.

Ovaries were dissected free and either fixed for immunohistochemical analysis, snap frozen and stored at  $-70^{\circ}\text{C}$ , placed in 500  $\mu\text{l}$  RNAlater (Ambion (Europe) Ltd, Huntingdon, Cambs, UK) or further dissected and placed into culture. Fixation was carried out in Bouin's fluid for 5 h, followed by transfer to 70% ethanol before processing into paraffin using standard methods.

### *Isolation of RNA and synthesis and amplification of cDNA*

Total RNA was extracted from fetal ovary (14–19 weeks) using either the Rneasy Mini Kit (Qiagen, Crawley, UK) for RT-PCR or TRIReagent (Sigma, Poole, Dorset, UK) for quantitative PCR analysis according to the manufacturers' instructions, and treated with DNase (Gibco, Paisley, UK). Reverse transcription using a first-strand cDNA synthesis kit (Roche Diagnostics, Lewes, UK) was followed by PCR using 1  $\mu\text{l}$  cDNA samples and Taq DNA polymerase (AGS Gold; Hybaid, Ashford, UK) as previously described (Robinson et al., 2001). Primers were designed to span an intron to ensure that genomic DNA was not amplified. Specific primers for each subunit and receptor are given in Table 1. Control tubes were run in parallel for each PCR, one in which water replaced RNA and a second RT-sample in which reverse transcriptase was omitted, to ensure there was no

Table 1  
Sequences of PCR primers

Primers	5'	3'
ActRIIA	GCAAAATGAATACGAAGTCTA	GCACCCTCTAATACCTCTGGA
ActRIIB	CAACTTCTGCAACGAGCGCTT	GCGCCCCGAGCCTTGATCTC
Inhibin $\alpha$	TGAGGGCCCTGTTCTTGGATG	CTGGCGGCTGCGTGATGCTG
Inhibin/activin $\beta$ A	GAACCTATGGAGCAGACCTCGG	TTGCCATCACACTCCAAGCC
Inhibin/activin $\beta$ B	GCCAGGAGCGCGTTTCCGAAATC	CCGCTCGCCCCGCTCAAACAAG
GAPD	GACATCAAGAAGGTGGTGAAGC	GTCCACACCCTGTTGCTGTAG

genomic DNA contamination. The identity of all PCR products were confirmed by direct sequencing using an Applied Biosystems 373A automated sequencer.

#### Lightcycler quantitative PCR

Quantitative PCR was performed using the Lightcycler system (Roche Molecular Biochemicals, Sussex, UK) as previously described (Hartley et al., 2002) to investigate changes in expression of the activin  $\beta$ A subunit over a range of gestations. Reverse transcribed RNA samples ( $n = 29$ ) were diluted in water as indicated. One microliter of diluted first-strand cDNA was added to a final volume of 10  $\mu$ l containing 2 mM  $MgCl_2$  and 0.5  $\mu$ M each of forward and reverse primer in  $1 \times$  Lightcycler Fast Start DNA MasterSYBR Green 1 Master Mix (Roche Molecular Biochemicals). Signal acquisition was performed for each of 45 amplification cycles followed by continuous melt curve analysis to ensure product accuracy. Primers for GAPD and activin  $\beta$ A subunit are given in Table 1.

Standard curves for GAPD and activin  $\beta$ A were derived by making a series of first-strand cDNA dilutions from a 16-week ovary. The number of cycles needed to yield a fluorescent signal above background (the cross-over point,  $C_p$ ) was plotted against the log of relative concentration using LightCycler Software (Molecular Dynamics Ltd., Buckinghamshire, UK) yielding a straight line for each product. Quantification of ovarian  $\beta$ A mRNA expression was subsequently performed. For each experiment, both GAPD and activin  $\beta$ A amplification reactions were performed in duplicate for every cDNA sample used. Calculations for activin  $\beta$ A mRNA concentration were made relative to GAPD from the same sample to allow comparisons between ovaries. Allowance for differences in amplification rate for GAPD and activin  $\beta$ A was achieved by determining the actual amount of amplification required to yield a signal for each target. Data were analysed by ANOVA.

#### Immunohistochemistry

Sections (5  $\mu$ m) were mounted on BDH Superfrost Plus slides (BDH Laboratory Supplies, Merck Ltd, Lutterworth, Leics, UK) and dried overnight at 50°C before processing for immunohistochemistry as previously described (Anderson et al., 2002). Briefly, slides were incubated in 3%

$H_2O_2$  in methanol for 30 min to inhibit endogenous peroxidase activity. After rinsing in distilled water, slides were washed twice in Tris-buffered saline (TBS; 0.05 mol/l Tris, 0.85% NaCl, pH 7.6) for 5 min and blocked for 30 min in appropriate serum (Diagnostics Scotland, Carlisle, UK) diluted 1:5 in TBS containing 5% bovine serum albumin. Sections were then blocked with avidin and biotin (both from Vector, Peterborough, UK) with washes in TBS in between. Various primary antibodies were used. Rabbit antibodies to Activin receptor II (ActRIIA) and Activin receptor IIB (ActRIIB) were kindly donated by Dr. C-H Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden, and used at a concentration of 1:600 and 1:400, respectively. Rabbit antibodies to ALK2 and ALK4 were also kindly donated by Dr. C-H Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden, and both used at concentration 1:150. Goat antibody to betaglycan (Santa Cruz Biotechnology, CA, USA) was used at a dilution of 1:25. Mouse monoclonal primary antibodies to the inhibin/activin subunits included  $\alpha$  subunit (173.9k) used at a range of dilutions 1:1000 to 1:100,  $\beta$ A subunit (E4) at 1:1000 and  $\beta$ B subunit (12/13) at 1:100, all the gift of NP Groome. Monoclonal antibody directed against recombinant follistatin FS288, which recognises follistatin even when bound to activin, was used at a range of dilutions 1:200 to 1:25, also gift of NP Groome (Majdic et al., 1997). All were incubated at 4°C overnight. Sections were then washed and incubated for 30 min with goat anti-rabbit (Santa Cruz Biotechnology), rabbit anti-goat (Dako, Cambridge, UK) or rabbit anti-mouse (Diagnostics Scotland) biotinylated secondary antibody as appropriate, at a dilution of 1:500. Following washes in TBS, sections were incubated with avidin-biotin-horseradish peroxidase linked complex (Dako) according to the manufacturer's instructions. Bound antibody was visualised using 3,3'-diaminobenzidine tetrahydrochloride (Dako). Nonimmune serum was used in place of primary antibody for negative controls. Positive controls (human fetal testis for activin/inhibin subunits and receptors and adult rat testis for follistatin) were also run in parallel.

Sections were counterstained with haematoxylin, dehydrated, mounted and visualised by light microscopy. Images were captured using an Olympus Provis microscope (Olympus Optical Co., London) equipped with Kodak DCS330 camera (Eastman Kodak Co, Rochester, NY).



### *Nuclear diameter measurement*

The size distribution of germ cells expressing activin  $\beta$ A subunit was determined in specimens of 18–19 weeks gestation. To avoid bias, sequential nonoverlapping frames were captured using the Provis image analysis system, and the diameter of all germ cell nuclei within the frame measured. Strongly immunostained germ cells were classified as  $\beta$ A positive, all other germ cells as negative. Data were analysed by the Mann–Whitney *U* test as the distribution deviated from normal. For graphical representation, numbers of immunopositive and immunonegative cells were grouped in 1- $\mu$ m increments of nuclear diameter. The nuclear diameter of a total of 527 germ cells was measured.

### *Primary cell culture*

Fetal ovaries were dissected and cultured in MEM $\alpha$  medium with phenol red (GibcoBRL, Life Technologies) supplemented with 2 mM pyruvate, 2 mM glutamine, 1 $\times$  ITS supplement (Sigma) and 3 mg/ml BSA, and with penicillin, streptomycin and amphotericin. A total of seven ovaries were used for culture experiments. Fetal ovaries (14–17 weeks) were dissected in prewarmed and gassed medium to yield tissue fragments approximately 1 mm<sup>3</sup>. Between four and six fragments were transferred in 400- $\mu$ l medium to each of four designated wells containing inserts (Millicell-CM; Millipore UK Ltd, Watford, UK) in a 24-well tissue culture plate such that tissue fragments were supported at the meniscus of the medium. Tissue was cultured for 17 h in a humidified incubator at 37°C and 5% CO<sub>2</sub>. Two treatment groups were cultured with 100 ng/ml recombinant human activin A (R&D systems, Abingdon, UK). The other two groups acted as controls. Bromodeoxyuridine (BrdU; Sigma) was supplemented at 10  $\mu$ l/ml as a marker of cell proliferation. In each experiment, BrdU was present in the media of one control and one treatment group for the duration of the experiment. BrdU was added to the other control and treatment group for the final 4 h of tissue culture only. Analysis of germ cell number in these parallel cultures allowed confirmation that changing the medium 4 h before the end of the culture period did not influence the results. At the end of culture, tissues were washed in PBS and fixed in Bouin's fluid for 1 h, followed by processing to visualise BrdU incorporation by immunohistochemistry.

Serial sections (5  $\mu$ m), four sections per slide, were mounted on BDH Superfrost Plus slides (BDH Laboratory Supplies) and dried overnight at 50°C. Every fourth slide was processed for immunohistochemistry as described above. The primary antibody used was formalin-grade anti-BrdU mouse monoclonal antibody (Roche) at a dilution of 1:30, incubated at 4°C overnight. On completion of the immunohistochemistry protocol, sections were counterstained with haematoxylin, dehydrated and mounted before analysis.

Sections of uncultured control and cultured tissue were analysed to quantify the effects of culture and activin A on the number and proliferation of germ cells. Analysis was carried out blind using the Area Fraction Probe in the Stereologer software programme (Systems Planning and Analysis Inc, Alexandria, VA, USA) as previously described (Robinson et al., 2003; Sharpe et al., 2002). Tissue was serially sectioned and sections greater than 20  $\mu$ m apart were counted, ensuring the same cells were not counted more than once. The counting was performed using a 121 point grid in the eyepiece of the microscope. Each tissue fragment was outlined and frames randomly selected thereafter by the programme. The number of points lying outside the tissue in any grid was also recorded and the total cell numbers were corrected for this. Average number of germ cells per grid was calculated for each experimental condition, and data were analysed using paired *t* tests.

## **Results**

### *Expression of mRNAs for activin/inhibin subunits and receptors*

mRNA expression was detected by RT-PCR using RNA extracted from human fetal ovaries (Fig. 1). Amplicons of expected sizes corresponding to the inhibin  $\alpha$  (278 bp), inhibin/activin  $\beta$ A (274 bp) and  $\beta$ B (325 bp) subunits and the activin receptors ActRIIA (435 bp) and ActRIIB (283 bp) were amplified from cDNA derived from ovaries across 14–19 weeks gestation. Product identity was confirmed by direct sequencing.

### *Quantitative PCR*

Changes in the expression of the activin  $\beta$ A subunit mRNA between 14 and 19 weeks gestation were investigated by quantitative RT-PCR. There was an approximately 2-fold increase in the relative expression of the  $\beta$ A subunit mRNA expression between 14 and 19 weeks gestation ( $P < 0.001$ , Fig. 2). The increase in expression was not continuous across the gestational range examined, but mostly occurred between 17 and 19 weeks.

### *Immunohistochemistry*

#### *Inhibin/Activin subunits*

Expression of the  $\beta$ A inhibin/activin subunit protein was detected in the fetal ovary at all gestations investigated. It was specifically localised to the germ cell cytoplasm at all gestations studied, but a marked change in the pattern of immunostaining was seen across the gestational range (Figs. 3D–F). At 14 weeks gestation, a gradient of distribution was observed. Oogonial immunostaining was detected throughout the ovary, but was most marked at the periphery, particularly in germ cells in the outermost few layers of the

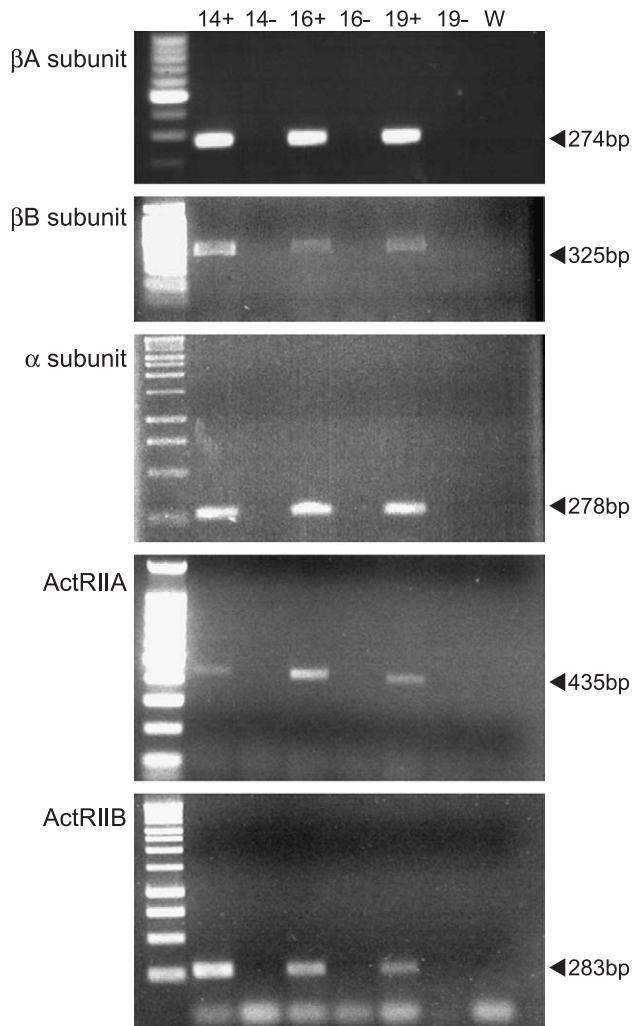


Fig. 1. Expression of mRNA for inhibin/activin subunits and type II activin receptors in the human fetal ovary. RT-PCR analysis of samples extracted from human fetal ovaries at 14, 16 and 19 weeks gestation. mRNA expression for inhibin/activin subunits and receptors ActRIIA and ActRIIB as indicated. Lanes marked 14–, 16– and 19– contained samples in which reverse transcriptase was omitted. W refers to water instead of cDNA in the PCR reaction. The size of each amplicon is indicated on the right.

gonad. At 17–19 weeks gestation, a different distribution of immunostaining had emerged with a discrete subpopulation of germ cells showing intense staining. These germ cells tended to be larger oocytes, and were distributed in small clusters of less than 10 largely located in the medullary region of the ovary. However, oocytes within newly formed primordial follicles showed only weak or no immunostaining for activin  $\beta$ A.

Differential expression of  $\beta$ A subunit by larger germ cells was confirmed by direct measurement of nuclear diameter. A total of 527 germ cells were measured, of which 79 (15%) were intensely immunopositive for the  $\beta$ A subunit. These  $\beta$ A-expressing germ cells were significantly larger than those only weakly immunopositive or immunonegative ( $11.3 \pm 0.2$  vs.  $9.8 \pm 0.1 \mu\text{m}$ ,  $P = 0.0001$ , Fig. 4) although the very largest germ cells with nuclear

diameter  $>16 \mu\text{m}$ , that is, those within primordial follicles, were immunonegative.

Expression of  $\beta$ B inhibin/activin subunit protein was also detected throughout the ovary across the range of gestations examined. Immunostaining was widespread, with both germ cells and stromal cells expressing the  $\beta$ B subunit (Figs. 3G–I). However, at the periphery of the ovary, some germ and somatic cells did not express the  $\beta$ B subunit. There was no difference in distribution of immunostaining of either stromal or germ cells between 14 and 18 weeks gestation. Two antibodies directed against the  $\beta$ B subunit (C12/13 monoclonal antibody, gift of NP Groome, and  $\alpha$ -cyclic inhibin  $\beta$ B (80–112)-NH<sub>2</sub> PBL#197 rabbit polyclonal, gift of W Vale) were used, with similar results.

Immunostaining for the inhibin  $\alpha$  subunit was not detected in any cell type in the human fetal ovary across a range of gestations and despite using a wide range of antibody titres. Fetal testes were used as a positive control and showed immunostaining in both Leydig cells in the interstitium and Sertoli cells in the seminiferous tubules as previously described (Figs. 3A–C) (Anderson et al., 2002).

#### Activin and inhibin receptors

Expression of the type II receptor ActRIIA was detected at all gestations from 14 to 21 weeks and was localised specifically to germ cell cytoplasm (Figs. 5C, D). There was no immunostaining in stromal cells or the pre-granulosa

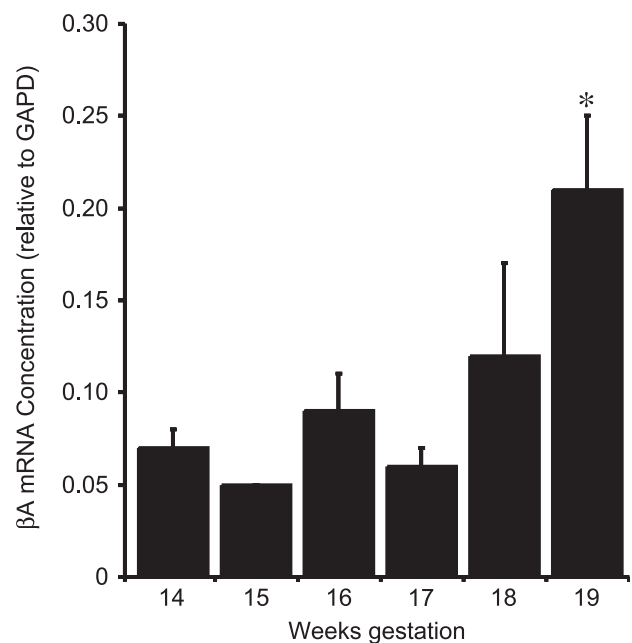


Fig. 2. Real-time PCR quantification of  $\beta$ A expression in the fetal ovary. Expression of  $\beta$ A subunit mRNA was quantified in human fetal ovary specimens over the gestational range 14–19 weeks. Data calculated as  $\beta$ A mRNA expression relative to GAPD for ovaries of 14, 15, 16, 17, 18 and 19 weeks gestation ( $n = 6, 5, 5, 5, 3$  and  $5$ , respectively, mean  $\pm$  SEM). SEM for 15 weeks = 0.00. \* $P < 0.001$  by ANOVA.

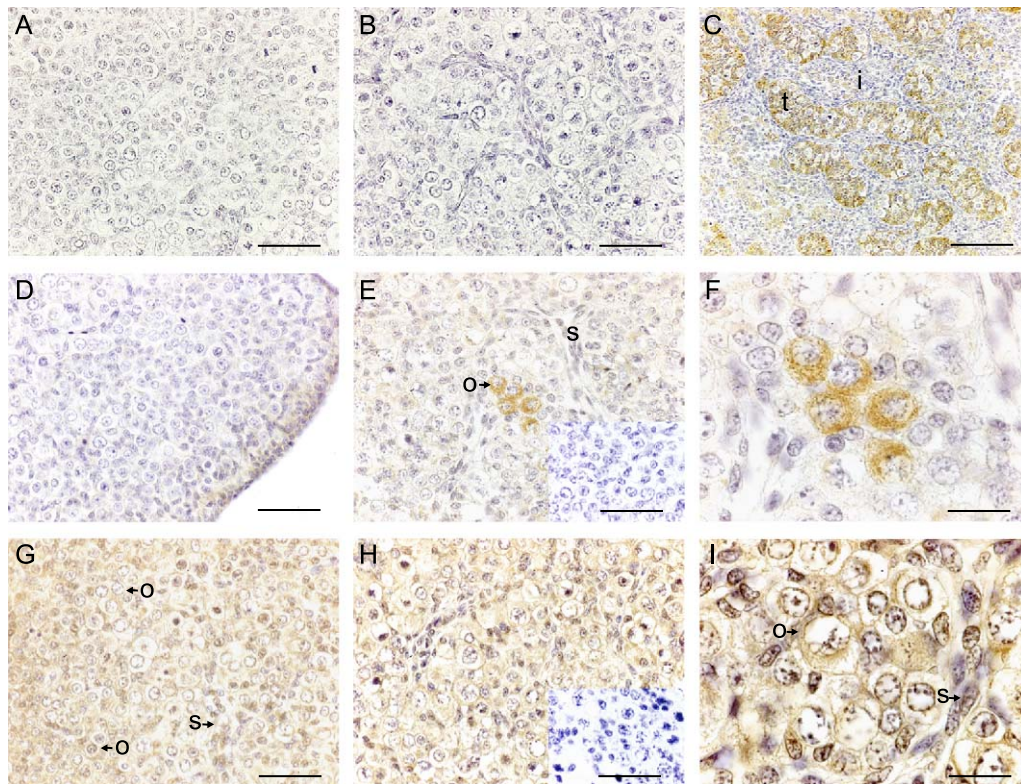


Fig. 3. Immunohistochemical localisation of inhibin/activin subunits in human fetal ovary. (A) Inhibin  $\alpha$  subunit, 14-week ovary. (B) Inhibin  $\alpha$  subunit, 19-week ovary. (C) Inhibin  $\alpha$  subunit positive control, 19-week testis. (D)  $\beta$ A subunit, 14-week ovary. (E)  $\beta$ A subunit, 17-week ovary (inset shows negative control). (F)  $\beta$ A subunit, 17-week ovary at higher magnification. (G)  $\beta$ B subunit, 14-week ovary. (H)  $\beta$ B subunit, 18-week ovary (inset shows negative control). (I)  $\beta$ B subunit, 18-week ovary at higher magnification. Positive staining in all panels is brown and sections are counterstained with haematoxylin. o, oogonia; s, stroma; t, tubule; i, interstitium. Scale bars in A–H, 50  $\mu$ m, F and I, 20  $\mu$ m.

cells of primordial follicles, nor cells at the ovarian surface. There was no significant difference in distribution across the gestational range studied.

ActRIIB immunoexpression was distributed widely within the fetal ovary at all gestations from 14 to 21 weeks (Figs. 5E, F). Immunostaining was detected in both stromal and germ cells but not in the cells at the ovarian surface. Intense immunostaining was noted in larger germ cells at later gestations and expression persisted in cytoplasm of oocytes organising into primordial follicles.

Expression of ALK4 protein, the preferential activin type I receptor, was detected throughout the ovary predominantly in stromal and pre-granulosa cells but also with weak expression in germ cells (Fig. 5A). At 14 weeks gestation, ALK4 immunostaining clearly demarcated a meshwork of ovarian stromal cells with weaker immunostaining of the oogonia intermingled with more strongly immunopositive pre-granulosa cells. At later gestations (18 weeks), the ovarian stroma was less strongly immunopositive, and the predominant site of ALK4 expression was the pre-granulosa cells surrounding individual and grouped oogonia, although oogonia remained weakly immunopositive.

In contrast to ALK4, expression of ALK2 protein was exclusively localised to the stromal cells at 14–18 weeks

gestation, with no immunostaining identified in germ cells at any gestation (Fig. 5B). Intense immunostaining was particularly notable in many pre-granulosa cells between and

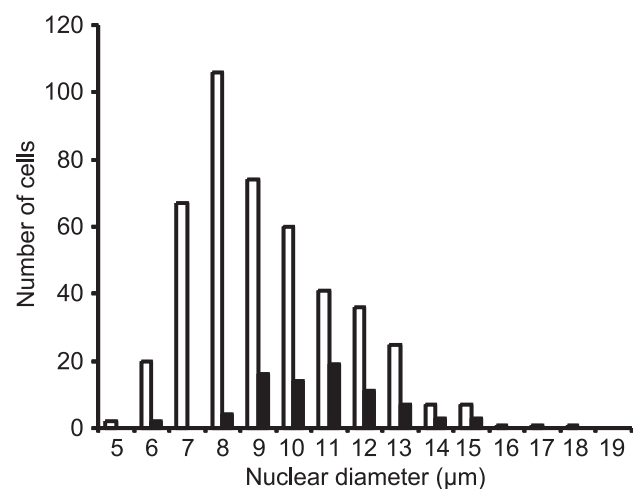


Fig. 4. Size distribution of  $\beta$ A-immunopositive germ cells in the human fetal ovary. Histogram shows the frequency of distribution of nuclear diameter of cells strongly immunopositive for  $\beta$ A subunit (filled columns) and those either weakly immunopositive or immunonegative for  $\beta$ A subunit (open columns) in human fetal ovary at 18–19 weeks gestation.  $n = 79$  and  $n = 448$ , respectively.



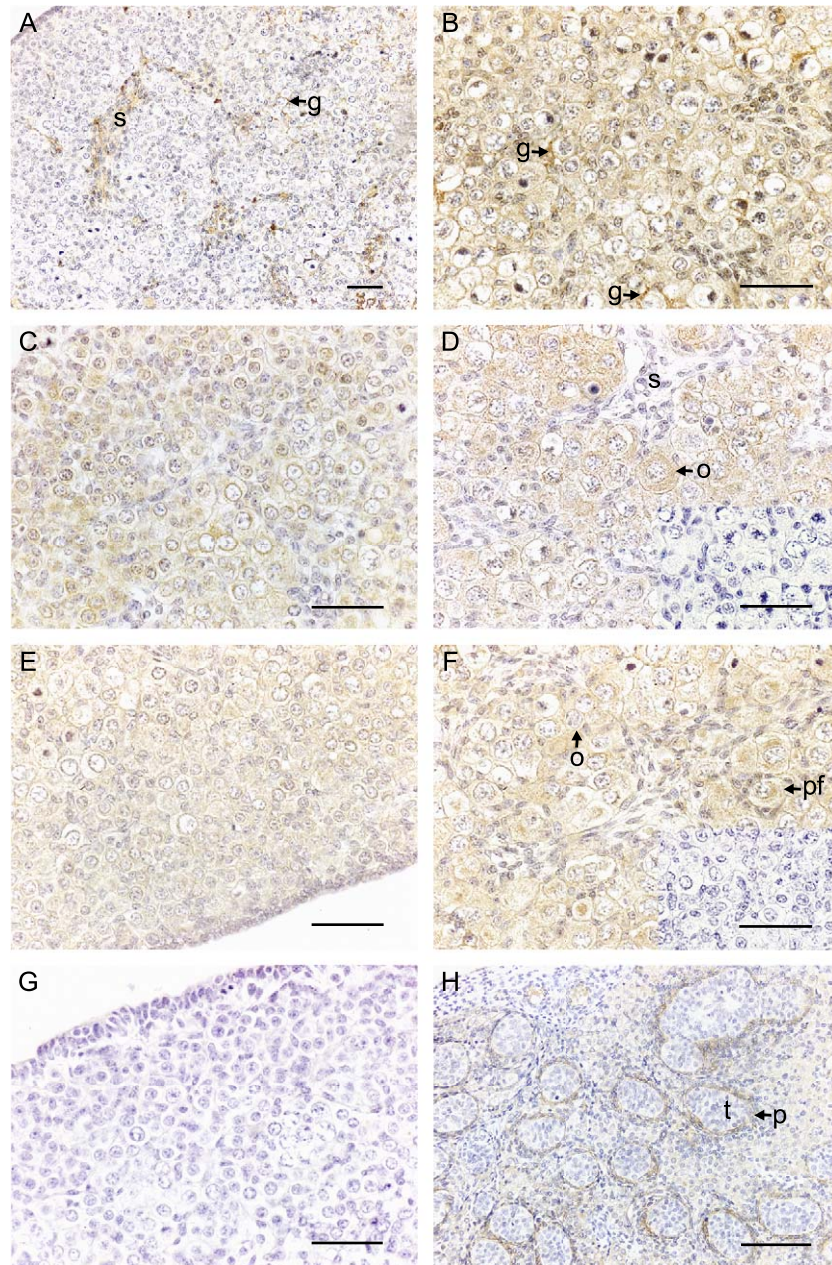


Fig. 5. Immunohistochemical localisation of inhibin/activin receptors in human fetal ovary. (A) ALK2, 14-week ovary. (B) ALK4, 18-week ovary. (C) ActRIIA, 14-week ovary. (D) ActRIIA, 21-week ovary (inset shows negative control). (E) ActRIIB, 14-week ovary. (F) ActRIIB, 21-week ovary (inset shows negative control). (G) Betaglycan, 14-week ovary. (H) Betaglycan-positive control, 15-week testis. Positive staining in all panels is brown and sections are counterstained with haematoxylin. s, stroma; g, pre-granulosa cells; o, oogonia; pf, primordial follicle; t, tubule; p, peritubular cells. Scale bars, 50  $\mu$ m in all panels.

outlining germ cells. This pattern of stromal cells immunostaining appeared to increase with increasing gestation and the formation of primordial follicles.

No expression of  $\beta$ -glycan protein was detected in any mid-trimester gestation ovary studied. Intense immunostaining was detected in the peritubular cells of fetal testes used as a positive control (Figs. 5G, H).

In all cases, negative controls showed no immunostaining. Fetal testis sections were used as a positive control,

and showed distinct immunostaining as previously described (Anderson et al., 2002).

#### *Follistatin*

No expression of follistatin was detected in any mid-trimester ovary studied (14–20 weeks gestation). Adult rat testis was used as a positive control and showed positive immunostaining in Leydig cells as previously described (Majdic et al., 1997).



## Tissue Culture

A total of seven separate experiments were carried out to investigate the effect of activin on germ cell survival and proliferation in vitro, using ovaries between 14 and 17 weeks gestation. Comparison of germ cell number in control cultures showed good oögonial survival ( $46 \pm 2$  oocytes per grid after 17 h culture vs.  $68 \pm 2$  in uncultured tissue) and there was no effect of changing the medium 4 h before the end of culture period in those experiments where BrdU was added at that time point ( $45 \pm 4$  germ cells per grid in controls vs.  $46 \pm 3$  in those in which the medium was changed). Treatment with 100 ng/ml activin A resulted in a significant increase in total number of germ cells. Activin A-treated groups showed a mean increase of 20% in the total number of germ cells from  $45 \pm 4$  to  $56 \pm 4$  per grid,  $P = 0.001$ . Very similar results were obtained in the cultures in which BrdU was present for 4 h (Fig. 6A). When BrdU was present in the culture medium for the duration of culture

(17 h), more germ cells were immunopositive than when BrdU was present for only the final 4 h of culture, as expected ( $6.4 \pm 1$  vs.  $2.8 \pm 0.4$  stained germ cells per grid when exposed to BrdU for only 4 h). Activin A treatment also resulted in a significant increase in number of BrdU-immunopositive germ cells. There was a 44% increase in number of immunostained germ cells ( $9.2 \pm 1.3$  vs.  $6.4 \pm 0.8$  per grid,  $P = 0.01$ ) when cultured with activin A and BrdU for 17 h. Immunostained germ cells were increased by 89% ( $5.3 \pm 0.9$  vs.  $2.8 \pm 0.4$  per grid,  $P < 0.05$ ) when cultured with activin A for 17 h and exposed to BrdU for only the final 4 h of culture (Figs. 6B, C).

## Discussion

High interspecies conservation of activins, inhibins and activin receptors and the universal presence of activins in mammals, birds, amphibians and fish suggest an evolution-

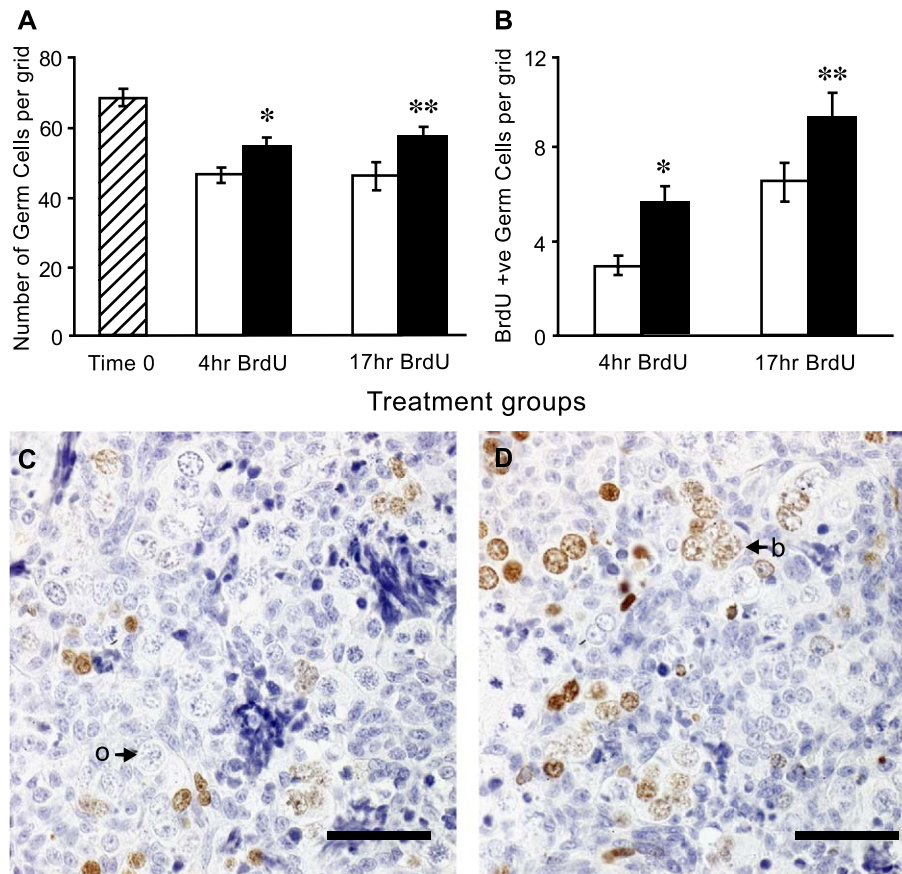


Fig. 6. Analysis of the effect of activin A on the number of germ cells in human fetal ovary. (A) Graph shows the number of germ cells per grid for time 0 tissue (shaded column), control groups (open columns) and groups cultured for 17 h with 100 ng/ml recombinant activin A (filled columns) in human fetal ovary at 14–17 weeks gestation (mean ± SEM). Treatment and control groups were either exposed to BrdU for 4 h or for the duration of culture (17 h) as indicated.  $*P < 0.05$ ;  $**P = 0.001$  vs. control. (B) Graph shows the number of BrdU-immunopositive germ cells per grid for control groups (open columns) and those cultured for 17 h with 100 ng/ml recombinant activin A (filled columns) in human fetal ovary at 14–17 weeks gestation (mean ± SEM). Treatment and control groups were either exposed to BrdU for 4 h or for the duration of culture (17 h).  $*P < 0.05$ ;  $**P = 0.01$  vs. control. Example of immunohistochemical staining of BrdU in cultured human fetal ovary. (C) Control 17-week ovary cultured for 17 h with BrdU. (D) Seventeen-week ovary cultured with 100 ng/ml recombinant activin A and BrdU for 17 h. Positive staining in all panels is brown and sections are counterstained with haematoxylin. o, oögonia; b, BrdU-immunopositive oögonia. Scale bar, 50 µm.

arily conserved role of these proteins in animal development. Activins have potent effects on early amphibian embryonic events during mesoderm-forming (Nieuwkoop) induction both in vitro and in vivo (Dyson and Gurdon, 1997). In mammals, activins have also been demonstrated to have morphogenetic effects, notably in renal tubule development (Ritvos et al., 1995). However, there are few published data available concerning the presence, expression and potential roles of activins, inhibins and their receptors during mammalian gonadal development. Whilst activin is thought to act primarily in an autocrine and/or paracrine manner, inhibin acts primarily as an endocrine feedback regulator of pituitary FSH release (Welt et al., 2002). Our data demonstrate the presence and localisation of activin receptors and subunits within the developing human ovary, and suggest a role for activin A in the regulation of germ cell proliferation during the developmental period leading up to primordial follicle formation. In contrast, follistatin was not expressed. Follistatin binds and neutralises activin activity. It also binds several other TGF $\beta$  superfamily members, including BMP4 (Fainsod et al., 1997; Lin et al., 2003). Follistatin expression has been detected in the fetal sheep ovary at late gestation, but not before primordial follicle formation (Braw-Tal et al., 1994). Similarly, follistatin is not expressed in the developing human or rat testis (Majdic et al., 1997) despite the presence of activin subunits and receptors (Anderson et al., 2002). It would therefore appear that activin activity in the developing human gonad is not regulated by follistatin.

Transgenic mice have been generated to study physiological models deficient in inhibins, activins and their receptors (Burns and Matzuk, 2002). Gene knockout experiments have been inconclusive in determining activin function in vivo, but functional analysis would suggest that activins are not essential for mammalian mesoderm formation. A number of reasons are cited to explain the lack of definitive conclusions from this body of work; firstly, some mutations are lethal, secondly, deficient activin ligand or receptor signalling results in a phenotype with suppressed FSH which results in reproductive and developmental defects and thirdly, there may be some functional overlap between TGF $\beta$  superfamily ligand and receptor signalling. Mutations in the gene encoding the  $\beta$ A subunit are lethal. Mutant mice develop to term but have secondary palate defects and lack whiskers and lower incisors (Matzuk et al., 1995b). The mutant mice die within 24 h of birth due to feeding difficulties and there are thus no conclusions regarding ovarian follicular development. Mice lacking the  $\beta$ B subunit suffer eye lesions as a result of failed eyelid fusion. Mutant females do not show a disruption in follicular development but manifest impaired reproduction characterised by perinatal lethality of their offspring (Vassalli et al., 1994). Highly increased  $\beta$ A expression was noted in the ovaries of these  $\beta$ B-deficient female mice, suggesting the possibility of functional compensation within the TGF $\beta$  superfamily. Using a gene knockin approach, it has been

found that activin  $\beta$ B can rescue the craniofacial defects and neonatal lethal phenotype of  $\beta$ A-deficient mice. However, activin  $\beta$ B is unable to substitute for all the functions of  $\beta$ A. The  $\beta$ B knockin mouse is subfertile and phenotypic abnormalities in the gonads and external genitalia, as well as delayed lethality remain in these mice. Ovaries in the hemizygous knockin female mouse are much smaller than those of controls (Brown et al., 2000). This may suggest activin  $\beta$ A expression is necessary for development of the correct complement of germ cells in the mouse ovary, consistent with the present data in the human. It is interesting that mice carrying a null mutation in the ActRII gene do not, as one might expect, phenocopy activin-deficient mice. Despite lacking a crucial component of the activin signalling pathway, only 25% of these mice die at or shortly after birth with mandible defects. Surviving adults have defective reproductive performance and small gonads although this may be in part due to abnormal FSH secretion in addition to deficient germ cell proliferation (Matzuk et al., 1995a). There is also some evidence that it is the second messenger (Smad) signalling which is more crucial to specific embryonic and extra-embryonic development pathways than particular ligand and receptor interactions (Chang et al., 2001).

Activin subunits and receptors showed a differential distribution within the developing ovary. Activins bind to a type II receptor (ActRIIA or ActRIIB), which recruits and phosphorylates a type I receptor with subsequent modulation of gene expression via Smad protein activation (Mas-sagué, 1998). Several type I receptors (ALKs) have been identified. ALK4 (ActRIB) is believed to be the preferential activin receptor. The activin  $\beta$ A subunit was specifically expressed by germ cells, whereas both somatic and germ cells expressed the  $\beta$ B subunit. ActRIIA was specifically localised to germ cells, whereas ActRIIB and the type I receptor ALK4 were expressed by both stromal and germ cells. The distinct distribution of both activin subunits and receptor subtypes between germ cells and somatic cells suggest that activin may have several distinct roles in ovarian development and organisation, and more specifically in germ cell regulation. This is supported by observations of an increase in  $\beta$ A mRNA expression as well as changes in the pattern of  $\beta$ A protein expression across the mid-trimester. With increasing gestation, intense  $\beta$ A expression was found in small clusters of larger, more mature germ cells. However, the largest oocytes, within primordial follicles, show little or no  $\beta$ A expression. This pattern suggests that the increase in  $\beta$ A subunit expression may be transient as the germ cells mature, with reduction of gene expression following primordial follicle formation. Increased expression however may be associated with differences in subsequent survival, consistent with the effects of activin on oogonial survival as demonstrated in the tissue culture experiments here described.

Despite detecting inhibin  $\alpha$  subunit mRNA by RT-PCR, we were unable to demonstrate the protein by immunohistochemistry. Previous studies suggest that activin but not

inhibin is present in the human fetal ovary at mid-gestation (16–23 weeks) (Rabinovici et al., 1991). Rabinovici et al. localised the  $\beta$ A subunit to primordial follicles by immunohistochemistry, but did not observe immunostaining for  $\beta$ B or  $\alpha$  subunits. In contrast, immunostaining for all three subunits was detected in late gestation fetal rhesus monkey ovary, which contained primordial and early growing follicles. Furthermore, culture medium from mid-gestation human fetal ovaries was found to contain undetectable levels of inhibin- $\alpha$  by radioimmunoassay. Recent immunolocalisation studies performed in baboons revealed minimal to nondetectable levels of  $\alpha$  subunit in mid- and late-gestation ovaries (Billiar et al., 2003) and there is also indirect evidence from *in situ* hybridisation studies on primate ovaries that follicles preferentially synthesise activin rather than inhibin in the early stages of development (Schwall et al., 1990; Yamoto et al., 1993). The putative inhibin receptor betaglycan (Lewis et al., 2000) was also not detected in the mid-trimester human fetal ovary. The fact that neither the  $\alpha$  subunit nor betaglycan receptor were identified by immunohistochemistry in mid-trimester human fetal ovaries indicates that inhibin is not a regulatory factor at this stage of development, but becomes of importance later during follicular growth (Welt et al., 2002).

An *in vitro* tissue culture model was used to investigate the effect of activin on oogonial survival and proliferation. Activin A treatment resulted in an increase in germ cell proliferation and survival in fetal ovary tissue fragments. This concurs with the earlier demonstration that activin A stimulated [ $^3$ H] thymidine incorporation *in vitro* in the differentiating rat ovary (Kaipia et al., 1994). BrdU was used as a marker for cell proliferation. It is interesting to note that immunostaining was observed predominantly towards the edge of cultured ovarian fragments. This may represent a diffusion effect, but may also partly reflect the organisation of the tissue. Mitotically dividing germ cells tend to be placed peripherally in the mid-trimester fetal ovary with a gradient of maturity across the developing ovary such that relatively more mature cells are found in the medulla compared to the cortex (Byskov, 1986). A recent study of follicle formation during fetal development in sheep also noted a cortical pattern of distribution of BrdU staining following *in vivo* administration (Sawyer et al., 2002), which became more pronounced up to primordial follicle formation.

In the adult ovary, activin A induces expression of genes signalling cellular differentiation and maturation. In the developing fetal ovary, key events involve germ cell proliferation, organisation and survival by somatic cell interaction and primordial follicle formation (Hirshfield, 1991; McLaren, 1991). The present data support a role of activin A in germ cell survival and proliferation. The Bcl-2 family of proteins are key regulators of apoptosis in many cell types. This family comprises pro-apoptotic (Bax, Bad and Bak) and anti-apoptotic (Bcl-2, Bcl-X<sub>L</sub> and Mcl-1) subfamilies. Mcl-1 is a rapidly inducible short-term effector of cell

viability and is expressed in a subset of oocytes at the time of primordial follicle formation (Hartley et al., 2002). The distribution of Mcl-1 in the fetal ovary is similar to that of activin  $\beta$ A subunit and it is possible that activin A promotes cell survival by signalling via Mcl-1, as has been demonstrated in a leukaemia cell line (Fukuchi et al., 2001). Whilst both activin A and Mcl-1 expression are most marked in larger oogonia preceding primordial follicle expression, oocytes within primordial follicles continue to express Mcl-1, whereas activin  $\beta$ A expression is low once that developmental stage is reached. It is possible that activin and Mcl-1 constitute part of the signalling pathway that determines survival for a small proportion of the oogonia in the mid-trimester ovary.

In conclusion, we have found the human fetal ovary to be a site of expression of activin subunits and receptors before the formation of primordial follicles. Protein expression of the inhibin  $\alpha$  subunit and the betaglycan receptor was not detected. The distribution of activin subunits and receptors suggests that whilst somatic cells may produce any of the activin forms, oogonia only produce activin A, and is consistent with an autocrine and/or paracrine mode of action for activins within the developing ovary. Increased expression of  $\beta$ A by larger/more mature oogonia may reflect selection for follicle formation and data from tissue culture experiments support activin A as a regulatory factor in fetal germ cell proliferation and survival. These data suggest that activin may be of particular importance during the period of development immediately preceding primordial follicle formation.

## Acknowledgments

We are grateful to Professor NP Groome, Oxford Brookes University for the inhibin/activin subunit and follistatin antibodies, to Professor W Vale, The Salk Institute, La Jolla, CA, USA, for the polyclonal  $\beta$ B antibody and to Dr. C-H Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden, for the ActRIIA, ActRIIB, ALK2 and ALK4 antisera.

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